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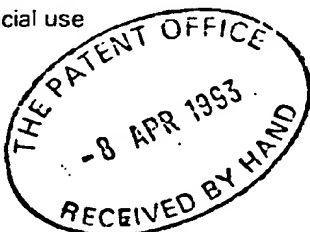
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Dated 23 May 2011

For official use



9307491.2

14 APR '93 H00402863 PAT 1 77 UC 25.00

Your reference

100-8024

08 APR 1993

Notes

Please type, or write in dark ink using CAPITAL letters. A prescribed fee is payable for a request for grant of a patent. For details, please contact the Patent Office (telephone 071-438 4700).

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**The
Patent
Office**

Request for grant of a Patent

Form 1/77

Patents Act 1977

1 Title of invention

- 1 Please give the title of the invention **ORGANIC COMPOUNDS.**

2 Applicant's details

☐ First or only applicant

- 2a If you are applying as a corporate body please give:

Corporate name **SANDOZ LTD.**

Country (and State of incorporation, if appropriate) **Switzerland**

- 2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

- 2c In all cases, please give the following details:

Address **35 Lichtstrasse, CH-4002 Basle, Switzerland**

UK postcode (if applicable)

Country **Switzerland**

ADP number (if known) **00703207001**

2d, 2e and 2f. If there are further applicants please provide details on a separate sheet of paper.

☐ **Second applicant (if any)**

2d If you are applying as a corporate body please give:

Corporate name

Country (and State
of incorporation, if
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Forenames

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(if known)

3 An address for service in the United Kingdom must be supplied

Please mark correct box

3 Address for service details

3a Have you appointed an agent to deal with your application?

Yes ☒ No ☐ → go to 3b

↓
please give details below

Agent's name B. A. YORKE & CO.

Agent's address Coomb House
7, St. John's Road
Isleworth
Middlesex TW7 0NH

Postcode

Agent's ADP number 1800001

3b: If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

Name

Address

Postcode

ADP number
(if known)

Daytime telephone
number (if available)

4 Agent's or
applicant's reference
number (if applicable) 100-3024

⑤ Claiming an earlier application date

Yes ☐ No ☒ **→ go to 6**

number of earlier application or patent number

 filing date

☐ and the Section of the Patents Act 1977 under which you are claiming:

15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

⑥ Declaration of priority

Country of filing

Priority application number
(if known)

Filing date
(day, month, year)

Please give the date in all number format, for example, 31/05/90 for 31 May 1990.

7 The answer must be 'No' if:

- any applicant is not an inventor
- there is an inventor who is not an applicant, or
- any applicant is a corporate body.

8 Please supply duplicates of claim(s), abstract, description and drawing(s).

Please mark correct box(es)

9 You or your appointed agent (see Rule 90 of the Patents Rules 1990) must sign this request

Please sign here ➡

A completed fee sheet should preferably accompany the fee.

6 Inventorship

7 Are you (the applicant or applicants) the sole inventor or the joint inventor?

Please mark correct box

Yes ☐

No ☒

A Statement of Inventorship on Patents Form 7/77 will need to be filed (see Rule 15).

8 Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application.

Continuation sheets for this Patents Form 1/77

no

Claim(s)

2

Description

10

Abstract

no

Drawing(s)

4

8b Which of the following documents also accompanies the application?

Priority documents (please state how many)

no

Translation(s) of Priority documents (please state how many)

no

Patents Form 7/77 – Statement of Inventorship and Right to Grant
(please state how many)

no

Patents Form 9/77 – Preliminary Examination/Search

no

Patents Form 10/77 – Request for Substantive Examination

no

9 Request

I/We request the grant of a patent on the basis of this application.

Signed SANDOZ LTD.

Date 07/04/1993

(day month year)

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ORGANIC COMPOUNDS

This invention relates to monoclonal antibodies to rapamycin, which are useful, e.g., in assay kits for monitoring blood levels of rapamycin.

Rapamycin is a macrolide antibiotic produced by Streptomyces hygroscopicus, which has been found to be pharmaceutically useful in a variety of applications, particularly as an immunosuppressant, e.g., for use in the treatment and prevention of organ transplant rejection and autoimmune diseases. Rapamycin, however, does exhibit side effects at higher dosages, and it has a somewhat variable bioavailability. Monitoring blood levels of rapamycin in patients being treated with rapamycin is thus very desirable in order to be able to regulate the dosage so as to maintain the minimum level sufficient for pharmacologic activity and to avoid any undue risk of pharmacologic activity. The lack of a sensitive and reliable assay which can be performed quickly and easily in a clinical setting has been a major obstacle to the development of rapamycin as a pharmaceutical.

Previous efforts to develop assay kits for clinical monitoring of rapamycin have not been particularly successful. EP 041795, for example, describes a microbiological assay in which rapamycin concentration is measured as a function of antifungal activity. WO 92/02946 provides an assay system which measures rapamycin levels indirectly by measuring competition for binding to macrophilin. Both of these assays are cumbersome

and not particularly sensitive. Even more importantly, both of these assays may have considerable variation under slightly different test conditions, making comparisons of test results from different hospitals difficult.

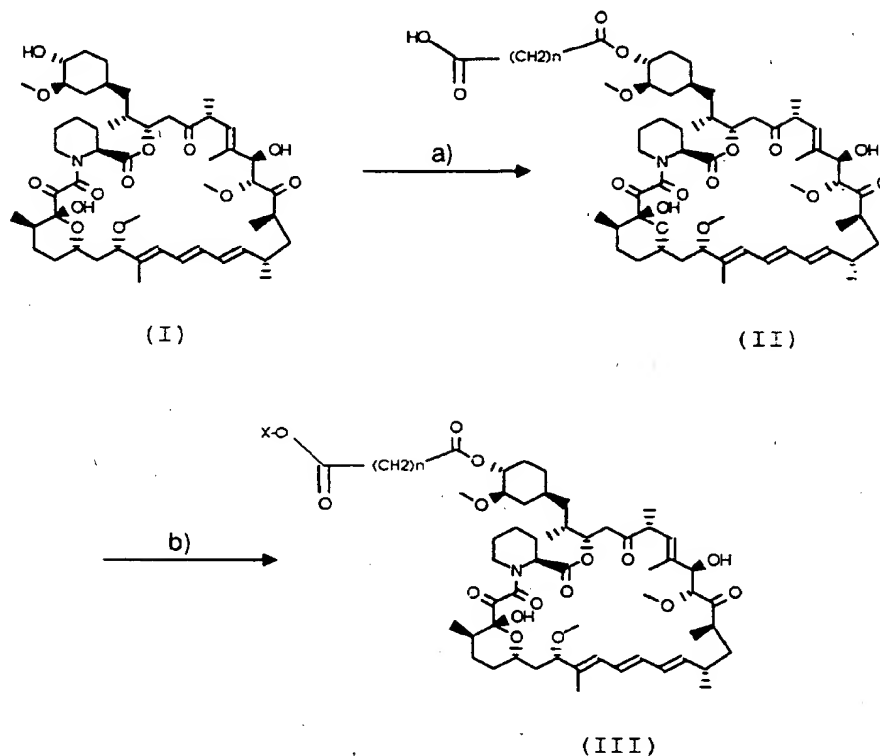
There have been no previous reports of monoclonal antibodies which are sensitive to rapamycin. There are inherent difficulties in making monoclonal antibodies to rapamycin because rapamycin is not immunogenic and is itself extremely immunosuppressive. Moreover, as the metabolites of rapamycin have not been well characterized in the literature, it is difficult to identify a monoclonal antibody capable of differentiating between rapamycin and its metabolites.

The present invention provides monoclonal antibodies which are highly sensitive to rapamycin. The antibodies of the invention are produced in response to inoculation with a novel immunogenic conjugate comprising a novel derivative of rapamycin linked to an immunogenic protein. Assay kits using these antibodies are well suited for use in a clinical setting and provide far more accurate and reproducible results than was previously possible.

The novel activated derivatives of rapamycin used to make the novel immunogenic conjugates of the invention are rapamycins which are linked through one of the hydroxy groups on rapamycin, preferably the hydroxy group located on the cyclohexyl portion of the rapamycin, to an activated coupling group, i.e., a group capable of direct reaction with a protein to form a covalent linkage without the requirement for the use of a coupling agent (e.g., carbodiimide reagents) to enable, effect, or promote the reaction with the protein. Cyclosporins bearing such activated coupling groups and their use in making immunogenic conjugates for the production of monoclonal antibodies to cyclosporin are discussed, e.g., in our European Patents 198 026 and 290 762 and our US patent 5,169,773, all incorporated herein by reference. Preferably, the activated coupling group has an activated ester or carboxy group, i.e., of formula $-CO-O-X$ where X is a carboxy activating group such as o- or p-nitrophenyl, 1-benztriazole, pentafluorophenyl, or

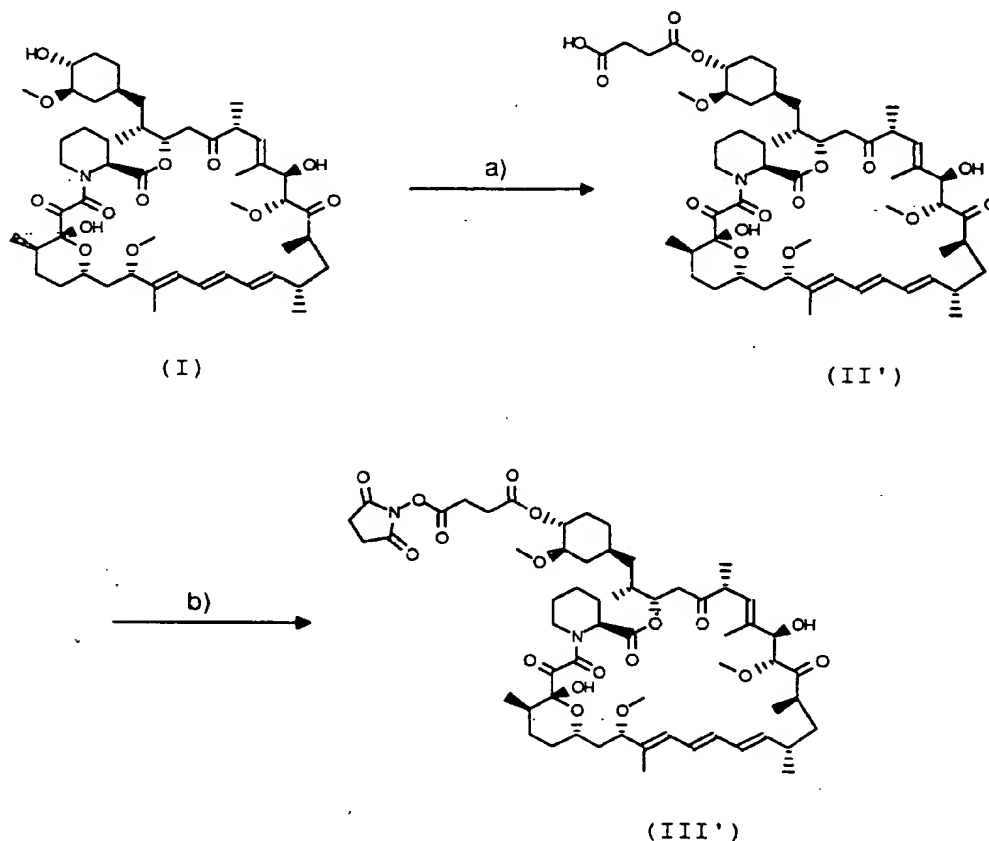
(especially) N-succinimido. Other suitable activated coupling groups are, for example, i) activated dithio groups, e.g., of formula -S-S-Z wherein X is a dithio activating group such as 2-pyridyl, which may be linked to the rapamycin; or ii) epoxy groups, e.g., epoxy methyl. The activated coupling group may be linked to the rapamycin by means of an ester, ether, amide, thio or other suitable linkage, but ester linkage is preferred. Most preferably, the activated coupling group contains a bis-ester moiety, e.g., succinyl, having an ester linkage to the rapamycin at one end and the activated ester or activated carboxy group at the other.

The preferred rapamycin derivatives of the invention are those of formula III below which are produced according to the following reaction scheme:



wherein formula I is rapamycin, which is a) reacted with an acylating agent, e.g., a cyclic anhydride under suitable conditions to yield a rapamycin of formula II having a free carboxy group. This rapamycin of formula II is then b) activated by reaction with a carboxy activating group, e.g. of formula $\text{HO}-\text{X}$ where X is as defined above, to yield the activated rapamycin of formula III.

The most preferred activated derivative of rapamycin is the succinimido derivative of formula III below, prepared as follows:



wherein formula I is Rapamycin, which is a) O-acylated using succinic anhydride in the presence of DMAP and pyridine to form the rapamycin hemisuccinate of formula II' (40-O-(3-Carboxy)propanoyl-rapamycin); which is then b) activated with N-hydroxy succinimide in the presence of EDC, Et₃N, and CH₂Cl₂ to form the succinimidooxysuccinyl rapamycin of formula III' (40-O-(3-Carboxy)propanoyl-rapamycin N-hydroxysuccinimide ester).

The activated rapamycins are then linked to suitable immunogenic proteins, e.g.,

bovine serum albumin (BSA), ovalbumin (OVA), or keyhole limpet hemocyanine (KLH) to form immunogenic conjugates. Monoclonal antibodies are prepared using conventional methods, e.g., administering the novel immunogenic conjugate to a suitable animal species to effect immunogenic challenge and recovering antibody-producing cells sensitized to said conjugate; immortalizing said antibody producing cells by fusion with a suitable myeloma; and recovering the monoclonal antibody from a selected immortalized cell line thus established.

The antibodies of the invention may then be used in a competitive assay measuring binding to a competitor, e.g., i) labeled (e.g., fluoro- or radio-labeled, especially biotinylated) rapamycin or ii) rapamycin-protein conjugate (e.g., the immunogenic conjugate described above), in the presence and absence of test fluid, e.g., plasma or whole blood from the patient. Optionally, either the antibody (in the first case) or the competitor (in the second case) are used to coat a microtiter plate. The assay is standardized with test solutions containing known concentrations of rapamycin. An assay kit comprising the monoclonal antibody of the invention, preferably in lyophilized form, and a competitor (and optionally further comprising a rapamycin solution for standardization and instructions for use) is capable of detecting rapamycin at concentrations of below 10 ng/ml, e.g., below 1 ng/ml, e.g., as low as 0.25-0.5 ng/ml.

Finally, the antibody of the invention may be further characterized by its relative binding affinity to FK-506. FK-506 is an immunosuppressive macrolide having some structural similarity to rapamycin in the binding domain. Both rapamycin and FK-506 bind to macrophilins (FKBPs), and for both it is believed that macrophilin binding is a necessary but not a sufficient criteria for immunosuppressive activity. The effector region of rapamycin, however, is quite different from FK-506, and indeed, the two compounds have quite different mechanisms of activity. (FK-506 for example appears to cause immunosuppression primarily by suppressing IL-2 transcription, whereas rapamycin has no significant effect on IL-2 transcription.) Rapamycin can thus be characterized as having an FKBP binding domain and an effector domain, and a distinction can be made between

rapamycin metabolites which are modified in the FKBP binding domain from those modified in the effector domain. This distinction can be made with the monoclonal antibodies of the invention by measuring the relative cross-reactivity of the monoclonal antibodies of the invention with FK-506: monoclonal antibodies having a high degree of cross reactivity (e.g., greater than 50%) recognize epitopes in the FKBP binding domain of rapamycin which is similar to FK-506; monoclonal antibodies with a low degree of cross reactivity (e.g., less than 20%, optimally less than 10%) recognize epitopes in the effector region, which is unique to rapamycin.

EXAMPLE 1- Production of activated derivative of rapamycin

a) Production of rapamycin hemisuccinate

To a stirred solution of 1.5 g (1.64 mmol) of rapamycin and 0.577 g (5.77 mmol) of succinic anhydride in 12 mL of pyridine is added 195 mg (1.64 mmol) of DMAP. The resulting mixture is stirred at ambient temperature for 19 h and concentrated under reduced pressure. The residue is dissolved in ethyl acetate and washed three times with water. The organic solution is dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue is purified by column chromatography on silica gel using 9:1 CH_2Cl_2 -MeOH. The fractions containing the expected product are combined and purified once more by column chromatography on silica gel using 19:1 CH_2Cl_2 -MeOH to afford, after removal of the solvent under reduced pressure, 40-O-(3-carboxy)propanoyl-rapamycin (the rapamycin hemisuccinate of formula II' supra) as a white foam having the following characteristic spectroscopic data:

^1H NMR (CDCl_3) δ 2.68 (7H, m, H33, H25 and $\text{O}_2\text{CCH}_2\text{CH}_2\text{CO}_2\text{H}$), 3.14 (3H, s and m, OCH_3 and H39), 3.34 (3H, s, OCH_3), 3.38 (3H, s, OCH_3), 4.68 (1H, m, H40), 4.72 (1H, broad s, 10-OH); MS (FAB) m/z 1036 ($[\text{M}+\text{Na}]^+$), 982 ($[\text{M}-\text{CH}_3\text{O}]^+$), 964 ($[\text{M}-(\text{CH}_3\text{O}+\text{H}_2\text{O})]^+$), 946 ($[\text{M}-(\text{CH}_3\text{O}+2\text{H}_2\text{O})]^+$).

b) Production of succinimidooxysuccinyl rapamycin

To a stirred solution of 120 mg (0.118 mmol) of the rapamycin hemisuccinate of step a), 16.5 mL (0.118 mmol) of Et_3N and 22.7 mg (0.118 mmol) of EDC in 8 mL of CH_2Cl_2 is added 13.6 mg (0.118 mmol) of N-hydroxysuccinimide. The resulting mixture is stirred for 18 h at room temperature, then diluted with ethyl acetate and washed twice with water. The organic solution is dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue is purified by column chromatography on silica gel (ethyl acetate) to afford 40-O-(3-Carboxy)propanoyl-rapamycin N-hydroxysuccinimide ester (the succinimidooxysuccinyl rapamycin of formula III' supra) as a white foam having the following characteristic spectroscopic data:

^1H NMR (DMSO) δ 2.57 (2H, t, $\text{O}_2\text{CCH}_2\text{CH}_2\text{CO}_2$), 2.81 (7H, s, CH_3O and succinimide CH_2), 2.92 (2H, t, $\text{O}_2\text{CCH}_2\text{CH}_2\text{CO}_2$), 4.55 (1H, m, H40), 5.26 (1H, d, 28-OH), 6.43 (1H, s, 10-OH); MS (FAB) m/z 1133 ($[\text{M}+\text{Na}]^+$), 1111 ($[\text{M}+\text{H}]^+$), 1092 ($[\text{M}-\text{H}_2\text{O}]^+$), 1079 ($[\text{M}-\text{CH}_3\text{O}]^+$), 1061 ($[\text{M}-(\text{CH}_3\text{O}+\text{H}_2\text{O})]^+$), 1043 ($[\text{M}-(\text{CH}_3\text{O}+2\text{H}_2\text{O})]^+$).

EXAMPLE 2 - Production of immunogenic conjugates

17.4 mg of the product of example 1 is dissolved in 400 μl DMF. 120 μl (5.22 mg rapamycin derivative) of this solution is added dropwise with vigorous stirring to a solution containing 8 mg of KLH in 2 ml 0.1 M NaHCO_3 buffer (pH 7.7). The reaction mixture is stirred for 2 hours at room temperature, and the resulting rapamycin-KLH conjugate is purified by dialysis at 4°C against 5 l PBS, 3X over 48 hours. The conjugate is optionally further concentrated by centrifugation using microconcentrator tubes. Rapamycin-BSA and rapamycin-OVA conjugates are prepared in the same manner, substituting BSA or OVA respectively for KLH in the above procedure.

EXAMPLE 3 - Production of monoclonal antibody

Monoclonal antibody is produced by using conventional techniques, essentially as described by Köhler and Milstein in *Nature* 256: 49. Female Bálb/C mice (20-25 g) each receive 100 µg of the immunogenic conjugate of example 2 in 0.2 ml complete Freund adjuvant, administered by i.p. injection. After 2 weeks a second booster injection comprising 50 µg of the immunogenic conjugate emulsified in 0.2 ml of complete Freund adjuvant is administered, again by i.p. injection. The presence of antibodies reactive to the antigen in the animals' blood serum is confirmed by competitive ELISA as described in example 4 below. The mice may optionally be further selected for antibody to the effector region (low cross reactivity with FK-506) and to the FKBP binding region (high cross reactivity to FK-506). Figure 1, for example, shows titer curves for a mouse (M1) having a high level of antibody to the binding domain of rapamycin, and another mouse (M7) having relatively high levels of antibody to the effector domain. Mice displaying maximum blood serum levels of antibody receive another booster injection comprising 20 µg of antigen. Four days later, they are sacrificed and their spleen cells are isolated and fused with PAI-0 cells (publicly available) or other suitable myeloma line. The resulting hybridomae are cultured and selected using ELISA for expression of antibody having a high affinity to rapamycin.

EXAMPLE 4 - Enzyme linked immunosorbent assay (ELISA)

Microtiter plates are coated with rapamycin-BSA conjugate prepared according to example 2. The plates are incubated for a fixed period, e.g., 8 hours, at low temperature, e.g., 4°C, under pH neutral buffered conditions in the presence of the antibody to be screened. Level of bound antibody is measured by anti-mouse-IgG peroxidase conjugate with 3,3',5,5'-tetramethylbenzidine as the substrate. Standard curves to determine affinity to rapamycin may be prepared using solutions containing known concentrations of rapamycin (e.g., 1 to 140 ng/ml in blood serum). Figure 2, for example, shows a standard curve for our monoclonal antibody M7-91, demonstrating that that monoclonal antibody, which was selected as being highly specific for rapamycin, is capable of detecting rapamycin at levels as low as 0.25 ng/ml. Antibodies may be further characterized as

binding to the effector or FKBP binding domains of rapamycin by measuring cross reactivity with FK-506 in an analogous ELISA using microtiter plates coated with FK506-BSA conjugate, which can be prepared analogously to rapamycin-BSA conjugate. For example, a comparison of binding levels of 17 selected monoclonal antibodies in the rapamycin-BSA and FK-506 assays is shown in figure 3; cross reactivity as a percentage is shown in figure 4.

Once the desired antibody has been selected, the same ELISA is used to determine blood levels of rapamycin in patients. A kit provides, e.g., one or more selected antibodies in lyophilized form, rapamycin-BSA conjugate coated microtiter plates, a rapamycin standard, and instructions for use. Anti-mouse IgG-enzyme conjugate and substrate as described above may additionally be provided, or the customer may use the monoclonal in his own established ELISA system.

CLAIMS

1. A monoclonal antibody capable of specifically recognizing rapamycin.
2. A monoclonal antibody according to claim 1 capable of recognizing an epitope on the FKBP-binding portion of rapamycin.
3. A monoclonal antibody according to claim 1 capable of recognizing an epitope on the effector portion of rapamycin.
4. A monoclonal antibody according to any one of claims 1 through 3, obtained or obtainable by:
 - a) reacting a rapamycin having an activated coupling group with an immunogenic protein to produce an immunogenic conjugate,
 - b) administration of said immunogenic conjugate to an appropriate animal species to, effect immunogenic challenge and recovery of antibody-producing cells sensitized to said conjugate,
 - c) immortalization of said antibody-producing cells, and
 - d) recovery of monoclonal antibody from a selected immortalized cell line thus established.
5. An immunogenic conjugate comprising a rapamycin portion and a protein portion.
6. An immunogenic conjugate according to claim 5 which is produced by reacting a protein with a rapamycin derivative bearing an activated coupling group.

7. An immunogenic conjugate according to claim 6 wherein the rapamycin derivative is 40-O-(3-Carboxy)propanoyl-rapamycin N-hydroxysuccinimide ester.
8. A rapamycin having an activated coupling group.
9. 40-O-(3-Carboxy)propanoyl-rapamycin N-hydroxysuccinimide ester .
10. A hybridoma cell line producing a monoclonal antibody as defined in any one of claims 1 through 4.
11. An immunoassay kit or system for monitoring the blood levels of a rapamycin, comprising a monoclonal antibody as defined in any one of claims 1 through 4.

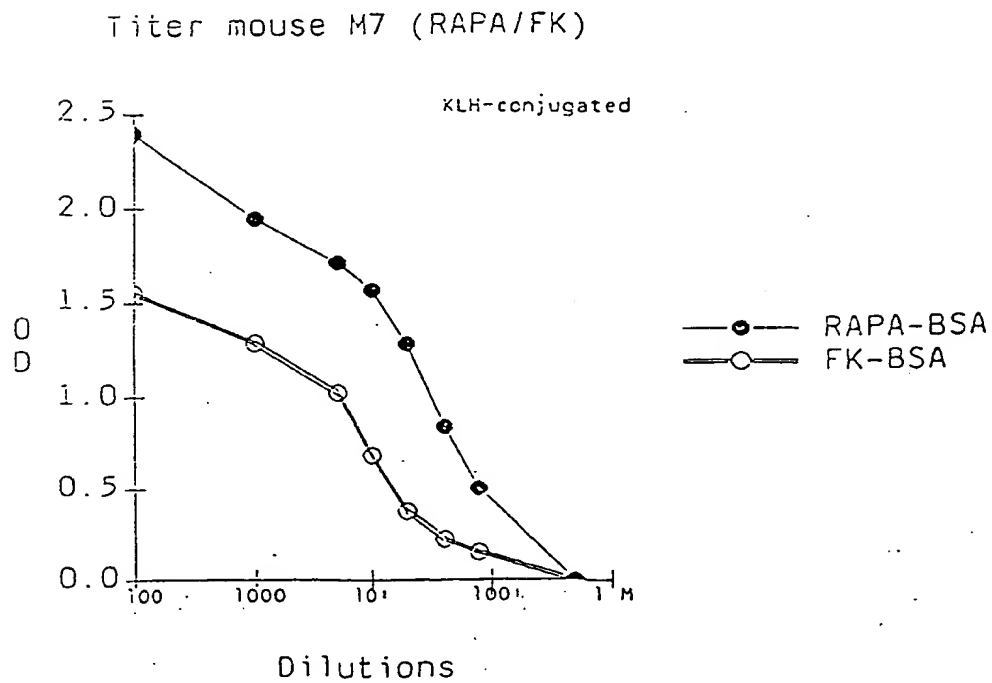
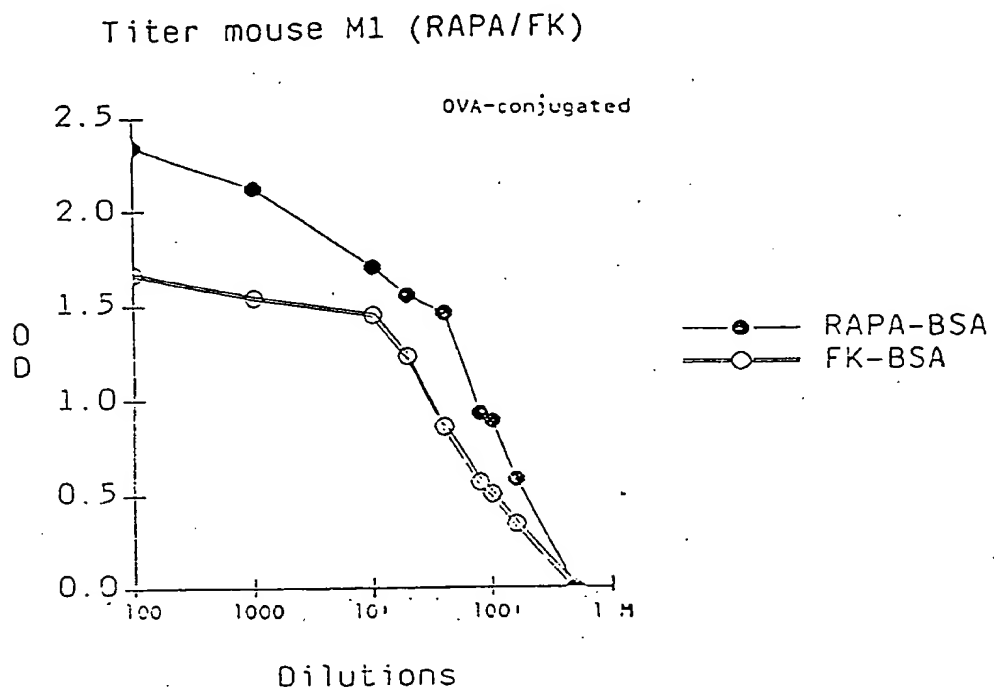


FIGURE 1

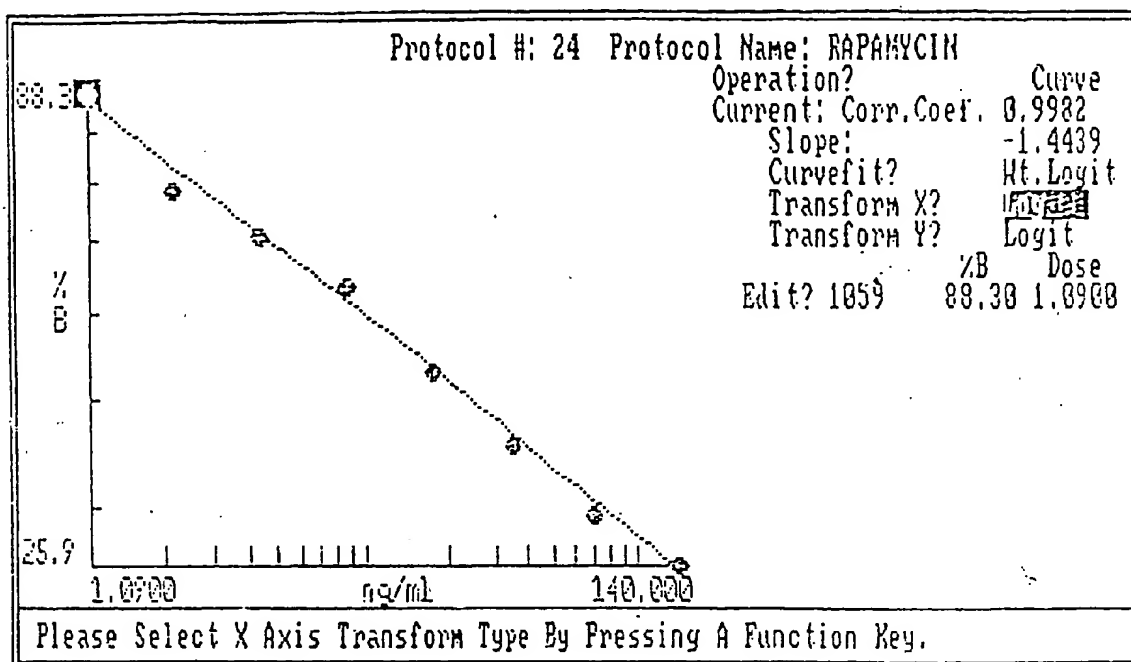


FIGURE 2

Screening of RAPA monoclonal antibodies

RAPA/FK 506 Elisa

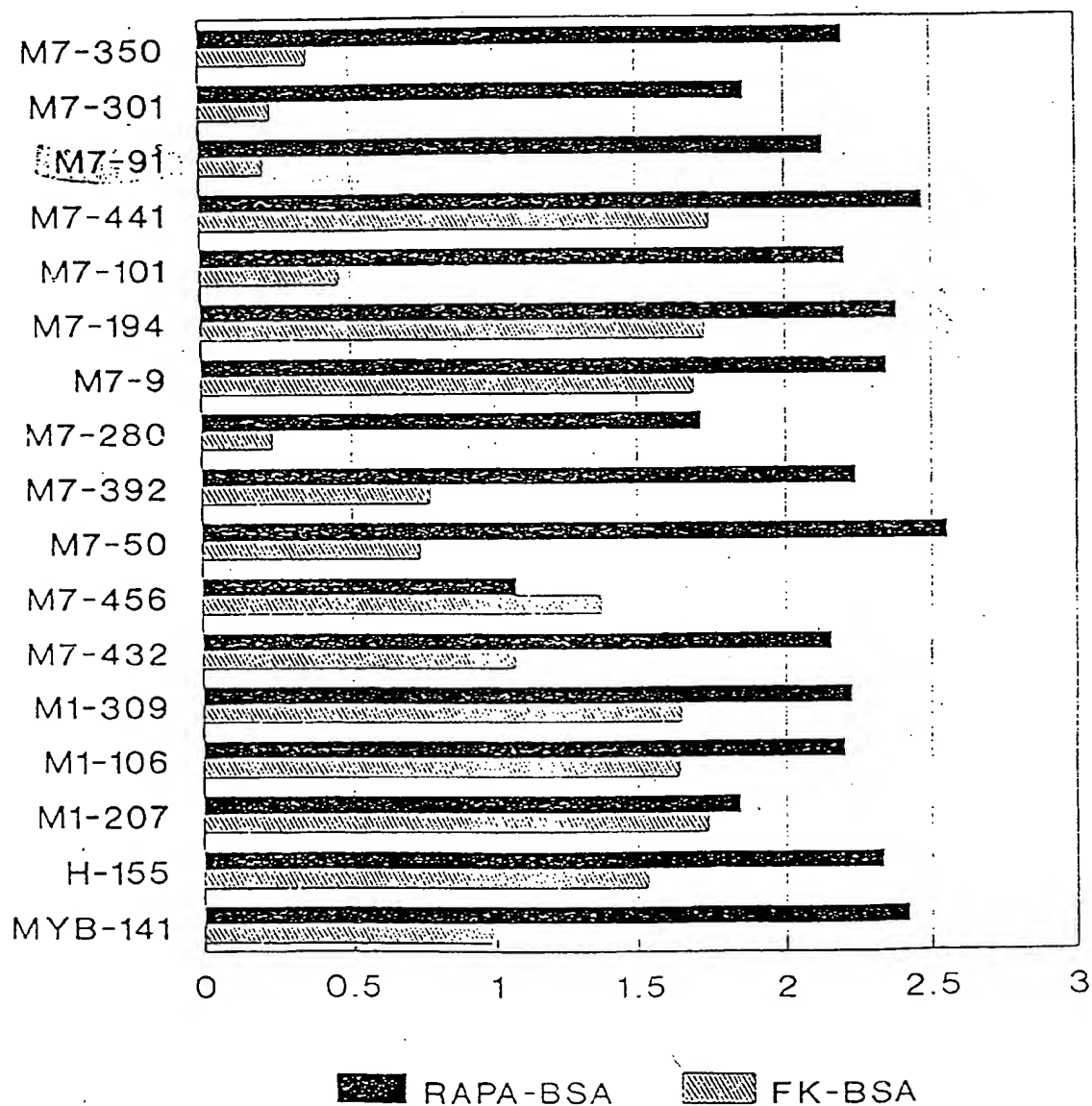


FIGURE 3

Screening of RAPA monoclonal antibodies

RAPA/FK 506 Elisa

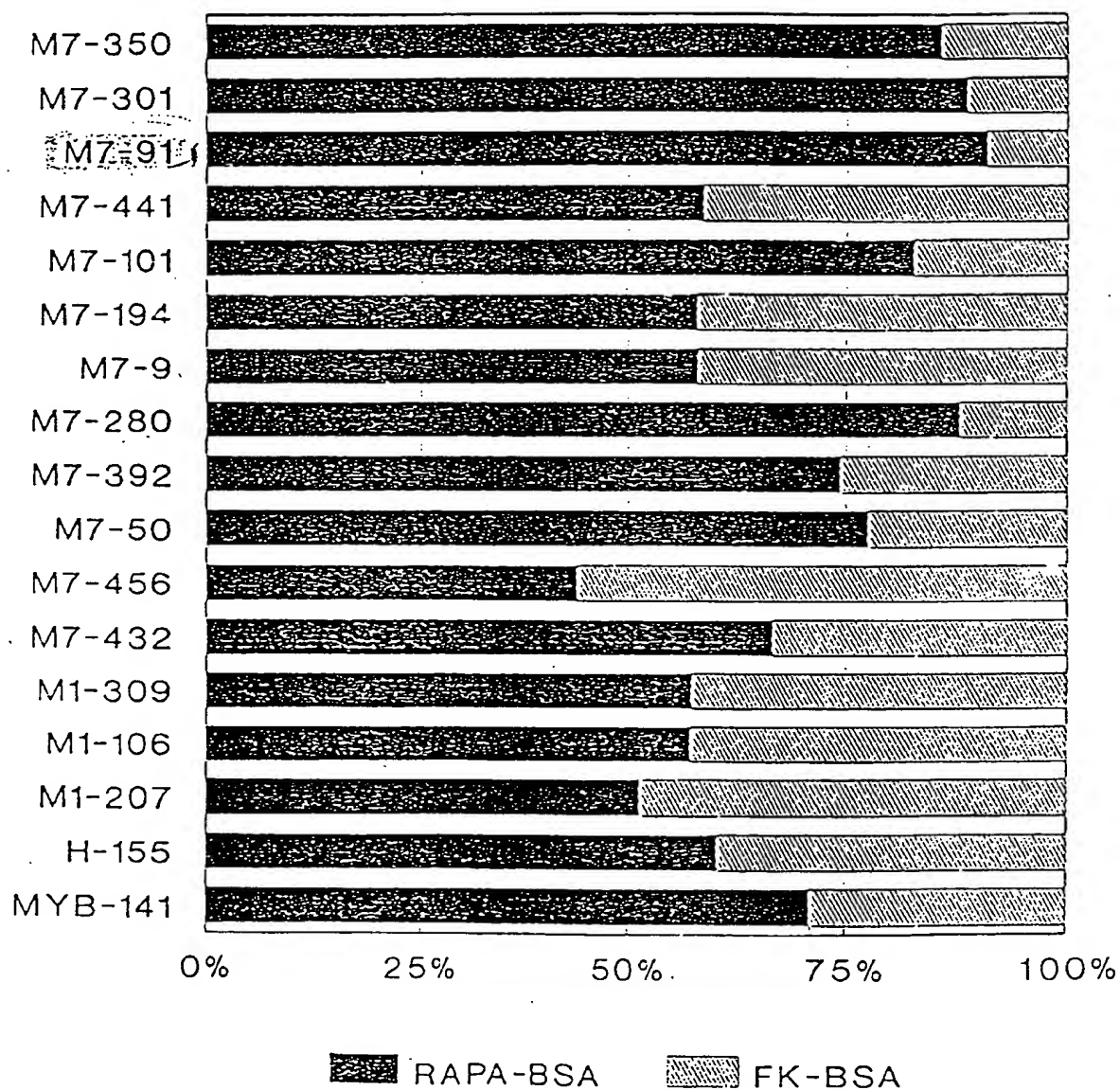


FIGURE 4